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(71) Applicants: **LUDWIG INSTITUTE OF CANCER RE-SEARCH** [US/US]; 605 Third Avenue, New York, NY 10158 (US). **LICENTIA LTD.** [FI/FI]; Erottajankatu 19 B 5, FIN-00130 Helsinki (FI).

(72) Inventors: **JELTSCH, Markku**; c/o Licentia Ltd., Erottajankatu 19 B 5, FIN-00130 Elsinki (FI). **ALITALO, Kari**; c/o Licentia Ltd., Erottajankatu 19 B 5, FIN-00130 Elsinki

(FI). **OLOFSSON, Birgitta**; University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0448 (FI). **ERIKSSON, Ulf**; c/o Ludwig Institute for Cancer Research, Box 240, S-17177 Stockholm (SE).

(74) Agent: **EVANS, Joseph, D.**; Crowell & Moring LLP, Intellectual Property Group, P.O. Box 14300, Washington, DC 20044-4300 (US).

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(54) Title: GLYCOSYLATED VEGF-B AND METHOD FOR INCREASING THE AMOUNT OF SOLUBLE VEGF-B

(57) Abstract: N-glycosylated VEGF-B proteins, nucleic molecule encoding these proteins, pharmaceutical compositions containing them and a method of increasing the amount of a soluble VEGF-B protein. The VEGF-B proteins are useful in stimulating and maintaining angiogenesis.

GLYCOSYLATED VEGF-B AND METHOD FOR
INCREASING THE AMOUNT OF SOLUBLE VEGF-B

BACKGROUND OF THE INVENTION

This invention relates to the discovery that N-glycosylation
of VEGF-B causes an increase in soluble proteins.

The two major components of the mammalian vascular system
5 are endothelial cells and smooth muscle cells. The endothelial
cells form the lining of the inner surface of all blood vessels
and lymphatic vessels in the mammal. The formation of new blood
vessels can occur by two different processes, vasculogenesis or
angiogenesis (for a review see Risau, W., Nature 386:671-674
10 (1997)). Vasculogenesis is characterized by the *in situ*
differentiation of endothelial cell precursors to mature
endothelial cells and association of these cells to form vessels,
such as occurs in the formation of the primary vascular plexus
in the early embryo. In contrast, angiogenesis, the formation
15 of blood vessels by growth and branching of pre-existing vessels,
is important in later embryogenesis and is responsible for most
of the blood vessel growth which occurs in the adult.
Angiogenesis is a physiologically complex process involving
proliferation of endothelial cells, degradation of extracellular
20 matrix, branching of vessels and subsequent cell adhesion events.
In the adult, angiogenesis is tightly controlled and limited
under normal circumstances to the female reproductive system.
However angiogenesis can be switched on in response to tissue
damage. Also solid tumors are able to induce angiogenesis in
25 surrounding tissue, thus sustaining tumor growth and facilitating
the formation of metastases (Folkman, J., Nature Med. 1:27-31,

(1995)).   The molecular mechanisms underlying the complex
angiogenic processes are far from being understood.

    Angiogenesis is also involved in a number of pathological
conditions, where it plays a role or is involved directly in
5 different sequelae of the disease.   Some examples include
neovascularization associated with various liver diseases,
neovascular sequelae of diabetes, neovascular sequelae to
hypertension,   neovascularization   in   post-trauma,
neovascularization due to head trauma, neovascularization in
10 chronic   liver   infection   (e.g.   chronic   hepatitis),
neovascularization due to heat or cold trauma, dysfunction
related to excess of hormone, creation of hemangiomas and
restenosis following angioplasty.   In arthritis, new capillaries
invade the joint and destroy cartilage.   In diabetes, new
15 capillaries in the retina invade the vitreous humour, causing
bleeding and blindness (Folkman, J. and Shing, Y., J. Biol. Chem.
267:10931-10934 (1992)). The role of angiogenic factors in these
and other diseases has not yet been clearly established.

    Because of the crucial role of angiogenesis in so many
20 physiological and pathological processes, factors involved in the
control of angiogenesis have been intensively investigated.   A
number of growth factors have been shown to be involved in the
regulation of angiogenesis.   These include fibroblast growth
factors   (FGFs),   platelet-derived   growth   factors   (PDGFs),
25 transforming growth factor alpha (TGF), and hepatocyte growth
factor (HGF).   See for example Folkman et al, J. Biol. Chem.,
267:10931-10934 (1992) for a review.

    It has been suggested that a particular family of
endothelial cell-specific growth factors known as the vascular
30 endothelial growth factors (VEGFs) and their corresponding
receptors are primarily responsible for stimulation of
endothelial cell growth and differentiation, and for certain
functions of the differentiated cells.   These factors are members

of the PDGF/VEGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs). The PDGF/VEGF family of growth factors belongs to the cystine-knot superfamily of growth factors, which also includes the neurotrophins and
5 transforming growth factor-ß.

Eight different proteins have been identified in the PDGF/VEGF family, namely two PDGFs (A and B), VEGF and five members that are closely related to VEGF. The five members closely related to VEGF are: VEGF-B, described in International
10 Patent Application No. WO 96/26736 and in U.S. Patent Nos. 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki; VEGF-C or VEGF2, described in Joukov et al, EMBO J. 15:290-298 (1996), Lee et al, Proc. Natl. Acad. Sci. USA 93:1988-1992 (1996), and U.S. Patents 5,932,540
15 and 5,935,540 by Human Genome Sciences, Inc; VEGF-D, described in International Patent Application No. PCT/US97/14696 (WO 98/07832), and Achen et al, Proc. Natl. Acad. Sci. USA 95:548-553 (1998); the placenta growth factor (PlGF), described in Maglione et al, Proc. Natl. Acad. Sci. USA 88:9267-9271 (1991); and VEGF3,
20 described in International Patent Application No. PCT/US95/07283 (WO 96/39421) by Human Genome Sciences, Inc. Each VEGF family member has between 30% and 45% amino acid sequence identity with VEGF in their VEGF homology domain (VHD). This VEGF homology domain contains the eight conserved cysteine residues which form
25 the cystine-knot motif. In their active, physiological state, the proteins are dimers. Functional characteristics of the VEGF family include varying degrees of mitogenicity for endothelial cells and related cell types, induction of vascular permeability and angiogenic and lymphangiogenic properties.
30 Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has been isolated from several sources. VEGF shows highly specific mitogenic activity for endothelial cells. VEGF has important regulatory functions in the formation of new

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blood vessels during embryonic vasculogenesis and in angiogenesis
during adult life (Carmeliet et al., Nature, 380: 435-439,
(1996); Ferrara et al., Nature, 380: 439-442, (1996); reviewed
in Ferrara and Davis-Smyth, Endocrine Rev., 18: 4-25, (1997)).
5 The significance of the role played by VEGF has been demonstrated
in studies showing that inactivation of a single VEGF allele
results in embryonic lethality due to failed development of the
vasculature (Carmeliet et al., Nature, 380: 435-439, (1996);
Ferrara et al., Nature, 380: 439-442, (1996)). The isolation and
10 properties of VEGF have been reviewed; see Ferrara et al., J.
Cellular Biochem., 47: 211-218, (1991) and Connolly, J. Cellular
Biochem., 47: 219-223, (1991).

In addition, VEGF has strong chemoattractant activity
towards monocytes, can induce the plasminogen activator and the
15 plasminogen activator inhibitor in endothelial cells, and can
also induce microvascular permeability. Because of the latter
activity, it is sometimes referred to as vascular permeability
factor (VPF). VEGF is also chemotactic for certain hematopoetic
cells. Recent literature indicates that VEGF blocks maturation
20 of dendritic cells and thereby reduces the effectiveness of the
immune response to tumors (many tumors secrete VEGF) (Gabrilovich
et al., Blood 92: 4150-4166, (1998); Gabrilovich et al., Clinical
Cancer Research 5: 2963-2970, (1999)).

Vascular endothelial growth factor B (VEGF-B) has similar
25 angiogenic and other properties to those of VEGF, but is
distributed and expressed in tissues differently from VEGF. In
particular, VEGF-B is very strongly expressed in heart, and only
weakly in lung, whereas the reverse is the case for VEGF
(Olofsson, B. et al, Proc. Natl. Acad. Sci. USA 93:2576-2581
30 (1996)). RT-PCR assays have demonstrated the presence of VEGF-B
mRNA in melanoma, normal skin, and muscle. This suggests that
VEGF and VEGF-B, despite the fact that they are co-expressed in
many tissues, may have functional differences. A comparison of

the PDGF/VEGF family of growth factors reveals that the 167 amino
acid isoform of VEGF-B is the only family member that is
completely devoid of any glycosylation. Gene targeting studies
have shown that VEGF-B deficiency results in mild cardiac
5 phenotype, and impaired coronary vasculature (Bellomo et al,
Circ. Res. 86:E29-35 (2000)).

Human VEGF-B was isolated using a yeast co-hybrid
interaction trap screening technique by screening for cellular
proteins which might interact with cellular retinoic acid-binding
10 protein type I (CRABP-I). The isolation and characteristics
including nucleotide and amino acid sequences for both the human
and mouse VEGF-B are described in detail in International
Application No. WO 96/26736 and in U.S. Patent Nos. 5,840,693 and
5,607,918 by Ludwig Institute for Cancer Research and The
15 University of Helsinki and in Olofsson et al, Proc. Natl. Acad.
Sci. USA 93:2576-2581 (1996). The nucleotide sequence for human
VEGF-B is also found at GenBank Accession No. U48801. The entire
disclosures of WO 96/26736, US 5,840,693 and US 5,607,918 are
incorporated herein by reference. The mouse and human genes for
20 VEGF-B are almost identical, and both span about 4 kb of DNA.
The genes are composed of seven exons and their exon-intron
organization resembles that of the VEGF and PlGF genes (Grimmond
et al, Genome Res. 6:124-131 (1996); Olofsson et al, J. Biol.
Chem. 271:19310-19317 (1996); Townson et al, Biochem. Biophys.
25 Res. Commun. 220:922-928 (1996)).

VEGF-C was isolated from conditioned media of the PC-3
prostate adenocarcinoma cell line (CRL1435) by screening for
ability of the medium to induce tyrosine phosphorylation of the
endothelial cell-specific receptor tyrosine kinase VEGFR-3
30 (Flt4), using cells transfected to express VEGFR-3. VEGF-C was
purified using affinity chromatography with recombinant VEGFR-3,
and was cloned from a PC-3 cDNA library. Its isolation and

characteristics are described in detail in Joukov et al., EMBO
J., 15: 290-298, (1996).

VEGF-D was isolated from a human breast cDNA library,
commercially available from Clontech, by screening with an
5 expressed sequence tag obtained from a human cDNA library
designated "Soares Breast 3NbHBst" as a hybridization probe
(Achen et al, Proc. Natl. Acad. Sci. USA, 95: 548-553, (1998)).
Its isolation and characteristics are described in detail in
International Patent Application No. WO98/07832 and in U.S.
10 Patent No. 6,235,713.   These documents also describe the
isolation of a biologically active fragment of VEGF-D which
consists of VEGF-D amino acid residues 93 to 201.   The VEGF-D
gene is broadly expressed in the adult human, but is certainly
not ubiquitously expressed.   VEGF-D is strongly expressed in
15 heart, lung and skeletal muscle. Intermediate levels of VEGF-D
are expressed in spleen, ovary, small intestine and colon, and
a lower expression occurs in kidney, pancreas, thymus, prostate
and testis.   No VEGF-D mRNA was detected in RNA from brain,
placenta, liver or peripheral blood leukocytes.

20   PlGF was isolated from a term placenta cDNA library. Its
isolation and characteristics are described in detail in Maglione
et al., Proc. Natl. Acad. Sci. USA, 88: 9267-9271, (1991).
Presently its biological function is not well understood.

VEGF3 was isolated from a cDNA library derived from colon
25 tissue.   VEGF3 is stated to have about 36% identity and 66%
similarity to VEGF.   The method of isolation of the gene encoding
VEGF3 is unclear and no characterization of the biological
activity is disclosed in International Patent Application No.
PCT/US95/07283 (WO 96/39421).

30   Similarity between two proteins is determined by comparing
the amino acid sequence and conserved amino acid substitutions
of one of the proteins to the sequence of the second protein,

whereas identity is determined without including the conserved amino acid substitutions.

As noted above, the PDGF/VEGF family members act primarily by binding to receptor tyrosine kinases. In general, receptor

5 tyrosine kinases are glycoproteins, which consist of an extracellular domain capable of binding a specific growth factor(s), a transmembrane domain, which is usually an alpha-helical portion of the protein, a juxtamembrane domain, which is where the receptor may be regulated by, e.g., protein

10 phosphorylation, a tyrosine kinase domain, which is the enzymatic component of the receptor and a carboxy-terminal tail, which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

Five endothelial cell-specific receptor tyrosine kinases

15 have been identified, belonging to two distinct subclasses: three vascular endothelial cell growth factor receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), and the two receptors of the Tie family, Tie and Tie-2 (Tek). These receptors differ in their specificity and affinity. All of them

20 have the intrinsic tyrosine kinase activity which is necessary for signal transduction.

The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds PlGF. VEGF-B binds to

25 VEGFR-1 with high affinity, but not to VEGFR-2 or -3 (Olofsson et al, Proc. Natl. Acad. Sci. USA, 95:11709-11714 (1998)). VEGF-C has been shown to be the ligand for VEGFR-3, and it also activates VEGFR-2 (Joukov et al, The EMBO Journal 15:290-298 (1996)). VEGF-D binds to both VEGFR-2 and VEGFR-3 (Achen et al,

30 Proc. Natl. Acad. Sci. USA 95:548-553 (1998)). A ligand for Tek/Tie-2 has been described in International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc. The ligand for Tie has not yet been identified.

A novel 130-135 kDa VEGF isoform specific receptor also has
been purified and cloned (Soker et al, Cell 92:735-745 (1998)).
The VEGF receptor was found to specifically bind the $VEGF_{165}$
isoform via the exon 7 encoded sequence, which shows weak
5 affinity for heparin (Soker et al, Cell 92:735-745 (1998)).
Surprisingly, the receptor was shown to be identical to human
neuropilin-1 (NP-1), a receptor involved in early stage
neuromorphogenesis. PlGF-2 also appears to interact with NP-1
(Migdal et al, J. Biol. Chem. 273:22272-22278 (1998)).

10   VEGFR-1, VEGFR-2 and VEGFR-3 are expressed differently by
endothelial cells. Generally, both VEGFR-1 and VEGFR-2 are
expressed in blood vessel endothelia (Oelrichs et al, Oncogene
8:11-18 (1992); Kaipainen et al, J. Exp. Med. 178:2077-2088
(1993); Dumont et al, Dev. Dyn. 203:80-92 (1995); Fong et al,
15 Dev. Dyn. 207:1-10 (1996)) and VEGFR-3 is mostly expressed in the
lymphatic endothelium of adult tissues (Kaipainen et al, Proc.
Natl. Acad. Sci. USA 9:3566-3570 (1995)). VEGFR-3 is also
expressed in the blood vasculature surrounding tumors.

Although VEGFR-1 is mainly expressed in endothelial cells
20 during development, it can also be found in hematopoetic
precursor cells during early stages of embryogenesis (Fong et al,
Nature 376:66-70 (1995)). In adults, monocytes and macrophages
also express this receptor (Barleon et al, Blood 87:3336-3343
(1995)). In embryos, VEGFR-1 is expressed by most, if not all,
25 vessels (Breier et al, Dev. Dyn. 204:228-239 (1995); Fong et al,
Dev. Dyn. 207:1-10 (1996)).

Since the identification and characterization of VEGF, a
number of important findings have focused attention on the
activity of angiogenic factors and the elucidation of new
30 factors. The early findings showed that angiogenesis is required
for normal development and physiology. Processes such as
embryogenesis, wound healing, and corpus luteum formation, all
involve angiogenesis and angiogenic factors. During wound

healing, for example, VEGF mRNA levels increase suggesting a direct correlation between the expression of VEGF and the healing process. Also, a defect in VEGF regulation might be associated with wound healing disorders (Frank, S., et al, J. Biol. Chem.

5   2705:12607-12613 (1995)).

Another important finding involves the connection between angiogenesis and tumor development. Both tumor growth and metastasis are angiogenesis-dependent processes (Folkman, J. and Shing, Y., J. Biol. Chem. 267: 10931-10934 (1992)). For example,

10   when tumor cells are introduced into an animal, the expression pattern of VEGF mRNA reveals expression at the highest level in cells at the periphery of necrotic, tumor growth areas. Numerous blood vessels were identified within these areas. The expression of VEGF in these areas suggests that hypoxemia, a state of

15   deficient oxygenation, triggers expression and release of VEGF in the necrotic tumor. The expression of VEGF-B also has been directly correlated with tumor growth (see U.S. Patent No. 5,840,693). VEGF-B expression is especially up regulated in tumor-associated macrophages and also in ovarian epithelial

20   tumors (Sowter et al, Lab Invest. 77:607-14, (1997)). VEGF-B mRNA can be detected in most tumor cell lines investigated, including adenocarcinoma, breast carcinoma, lymphoma, squamous cell carcinoma, melanoma, fibrosarcoma and Schwannoma (Salven et al, Am J Pathol. 153:103-8 (1998)).

25   It has been shown that members of the VEGF/PDGF family produce variant transcripts. VEGF has been shown to display different transcripts because of alternative splicing. The human VEGF gene has five different mRNA species (Neufeld et al, FASEB J. 13:9-22 (1999)), resulting in proteins differing in their

30   molecular mass and biological properties (Carmeliet, P., Nat. Med. 6:389-395 (2000)). The hVEGF-A_{165} isoform is the predominant transcript in most human tissues, giving rise to a polypeptide with affinity to the neuropilin-1 receptor, besides the binding

to VEGFR1 and VEGFR2. The $hVEGF_{121}$ and $hVEGF_{189}$ isoforms are expressed in normal tissues at lower levels. The $hVEGF_{206}$ isoform is mainly expressed in embryonic tissues (Houck et al, Mol Endocrinol. 5:1806-14 (1991)), while $hVEGF_{145}$ can only be found in

5 tumor cell lines (Poltorak et al, J Biol Chem. 272:7151-8 (1997)). Moreover, VEGF is also regulated in an isoform-specific way under pathological conditions. In lung and colon carcinomas, $hVEGF_{165}$ and $hVEGF_{121}$ are up-regulated, whereas $hVEGF_{189}$ is not changed, suggesting an isoform-specific role of VEGF in

10 malignancy (Cheung et al, Hum Pathol. 29:910-4 (1998)). An isoform specific VEGF targeting experiment with murine VEGF-B has shown that $mVEGF_{164}$ and $mVEGF_{188}$ are more important for postnatal growth and maintenance of normal function of cardiovascular system, while $mVEGF_{120}$ initiates and promotes vasculogenesis

15 (Carmeliet et al, Nat Med. 5:495-502 (1999)).

The placenta growth factor (PlGF) has three different isoforms, which are expressed in a tissue and development specific way (Maglione et al, Oncogene 8:925-31 (1993); Cao et al, Biochem Biophys Res Commun. 235:493-8 (1997)).

20 Two isoforms of VEGF-B, generated by alternative splicing of mRNA, have been recognized (Grimmond et al, Genome Res. 6:124-131 (1996); Olofsson et al, J. Biol. Chem. 271:19310-19317 (1996); Townson et al, Biochem. Biophys. Res. Commun. 220:922-928 (1996)). They are a cell associated form of 167 amino acid

25 residues ($VEGF-B_{167}$) and a secreted form of 186 amino acid residues ($VEGF-B_{186}$). The isoforms have an identical N-terminal domain of 115 amino acid residues, excluding the signal sequence. The common N-terminal domain is encoded by exons 1-5. Differential use of the remaining exons 6A, 6B and 7 gives rise

30 to the two splice isoforms. By the use of an alternative splice-acceptor site in exon 6, an insertion of 101 bp introduces a frame-shift and a stop of the coding region of $VEGF-B_{167}$ cDNA. Thus, the two VEGF-B isoforms have differing C-terminal domains.

The different C-terminal domains of the two splice isoforms
of VEGF-B affect their biochemical and cell biological
properties. The C-terminal domain of VEGF-B$_{167}$ is structurally
related to the corresponding region in VEGF, with several
5 conserved cysteine residues and stretches of basic amino acid
residues. Thus, this domain is highly hydrophilic and basic and,
accordingly, VEGF-B$_{167}$ will remain cell-associated on secretion,
unless the producing cells are treated with heparin or high salt
concentrations. The cell-associated molecules binding VEGF-B$_{167}$
10 are likely to be cell surface or pericellular heparin sulfate
proteoglycans. It is likely that the cell-association of this
isoform occurs via its unique basic C-terminal region.

The C-terminal domain of VEGF-B$_{186}$ has no significant
similarity with known amino acid sequences in the databases. The
15 hydrophobic character of the C-terminal domain of VEGF-B$_{186}$
contrasts with the properties of the hydrophilic and basic C-
terminal domain of VEGF-B$_{167}$. This is supported by the
observation that VEGF-B$_{186}$ does not remain cell-associated on its
secretion. Recent evidence indicates that this isoform is
20 proteolytically processed, which regulates the biological
properties of the protein (Olofsson et al, Proc. Natl. Acad. Sci.
USA, 95:11709-11714 (1998)).

A further difference is found in the glycosylation of the
VEGF-B isoforms. VEGF-B$_{167}$ is not glycosylated at all, whereas
25 VEGF-B$_{186}$ is O-glycosylated but not N-glycosylated.

Both isoforms of VEGF-B also form heterodimers with VEGF,
consistent with the conservation of the eight cysteine residues
involved in inter- and intramolecular disulfide bonding of PDGF-
like proteins. Furthermore, co-expression of VEGF-B and VEGF in
30 many tissues suggests that VEGF-B-VEGF heterodimers occur
naturally. Heterodimers of VEGF-B$_{167}$-VEGF remain cell-associated.
In contrast, heterodimers of VEGF-B$_{186}$ and VEGF are freely
secreted from cells in a culture medium. VEGF also forms

heterodimers with PlGF (DiSalvo, et al, J. Biol. Chem. 270:7717-7723 (1995)). The production of heterodimeric complexes between the members of this family of growth factors could provide a basis for a diverse array of angiogenic or regulatory molecules.

5   Since the secreted VEGF-B$_{167}$ remains cell-associated, it is intrinsically difficult to obtain significant amounts of soluble VEGF-B$_{167}$. Accordingly, there is a need to develop methods for increasing the amount of soluble VEGF-B$_{167}$.

10 SUMMARY OF THE INVENTION

This invention relates to a N-glycosylated VEGF-B and a method for increasing the amount of soluble VEGF-B proteins.

In a first aspect, the invention provides a purified and isolated nucleic acid molecule having a polynucleotide sequence

15 selected from the group consisting of SEQ ID NO:1 (sequence encoding VEGF-B$_{167}$), SEQ ID NO:3 (sequence encoding VEGF-B$_{186}$) and SEQ ID NO:5 (sequence encoding VEGF-B$_{Ex1-5}$) into which a nucleotide sequence encoding at least one putative N-glycosylation site has been inserted. The nucleic acid molecule having said

20 polynucleotide sequence can be naked and/or in a vector or liposome. The putative N-glycosylation site is -NXT-, -NXS- or -NXC-, where N represents the amino acid asparagine, X may be any amino acid, and T, S and C represent the amino acids threonine, serine and cysteine, respectively. The nucleotide sequence which

25 encodes the N-glycosylation site may thus comprise aay-nnn^1-(wgy/wcn)-nnn^2, with the proviso that -nnn^1- is not tga, tar or cnn, and -nnn^2- is preferably not ccn, where w represents adenine or thymine/uracil, g represents guanine, y represents cytosine or thymine/uracil, c represents cytosine, n represents adenine,

30 cytosine, guanine or thymine/uracil; t represents thymine/uracil, a represents adenine, and r represents guanine or adenine. (Rules   for   N-glycosylation   are   described   at

http://www.expasy.ch/cgi-bin/nicedoc.pl?PDOC00001). Preferably
the nucleotide sequence comprises aay-nnn^1-(agy/wcn)-nnn^2.

The invention includes the nucleic acid molecules described
above as well as fragments of those polynucleotides, and variants
5 of those polynucleotides with sufficient similarity to the non-
coding strand of those polynucleotides to hybridize thereto under
stringent conditions and which can code for VEGF-B or a fragment
or analog thereof which exhibits at least 90% sequence identity
to SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and which binds to
10 VEGFR-1. Thus, such polynucleotide fragments and variants having
a nucleotide sequence encoding at least one putative N-
glycosylation site inserted therein are intended as aspects of
the invention. Exemplary stringent hybridization conditions are
as follows: hybridization at 42°C in 5X SSC, 20 mM NaPO$_4$, pH 6.8,
15 50% formamide; and washing at 42°C in 0.2X SSC. Those skilled
in the art understand that it is desirable to vary these
conditions empirically based on the length and the GC nucleotide
base content of the sequences to be hybridized, and that well
accepted formulas for determining such variation exist. See for
20 example Sambrook et al, "Molecular Cloning: A Laboratory Manual",
Second Edition, pages 9.47-9.51, Cold Spring Harbor, New York:
Cold Spring Harbor Laboratory (1989).

Moreover, purified and isolated nucleic acid molecules
having a polynucleotide sequence encoding other, non-human,
25 mammalian VEGF-B forms and having a nucleotide sequence encoding
at least one putative N-glycosylation site inserted therein are
aspects of the invention, as are the polypeptides encoded
thereby.

A second aspect of the invention involves the purification
30 and isolation of a protein having an amino acid sequence selected
from the group consisting of SEQ ID NO:2 (VEGF-B$_{167}$), SEQ ID NO:4
(VEGF-B$_{186}$) and SEQ ID NO:6 (VEGF-B$_{Ex1-5}$) and having at least one
putative N-glycosylation site inserted therein. The purified and
isolated protein preferably is produced by the expression of the

nucleic acid molecule of the invention. As noted above, the at
least one putative N-glycosylation site is -NXT-, -NXS- or NXC,
where N represents the amino acid asparagine, X may be any amino
acid, and T, S and C represent the amino acids threonine, serine
5 and cysteine, respectively. Preferably the N-glycosylation site
is -NXT- or -NXS-, especially preferably -NXT-. It is also
preferred that X and the amino acid following T or S not be
proline.

As used herein, the term "VEGF-B" collectively refers to the
10 known VEGF-B167 and VEGF-B186 polypeptide isoforms as well as to
fragments or analogs thereof which exhibit at least 90% sequence
identity to SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and which
bind to VEGFR-1 and/or have the vasculogenesis stimulating
activity of VEGF-B.. The active substance preferably will include
15 the amino acid sequence Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-
Gly-Cys-Cys (where Xaa may be any amino acid) which is
characteristic of VEGF-B.

Polypeptides   comprising   conservative   substitutions,
insertions, or deletions, but which still retain the biological
20 activity of VEGF-B are clearly to be understood to be within the
scope of the invention. Persons skilled in the art will be well
aware of methods which can readily be used to generate such
polypeptides, for example the use of site-directed mutagenesis,
or specific enzymatic cleavage and ligation. The skilled person
25 will also be aware that peptidomimetic compounds or compounds in
which one or more amino acid residues are replaced by a non-
naturally occurring amino acid or an amino acid analog may retain
the required aspects of the biological activity of VEGF-B. Such
compounds can readily be made and tested by methods known in the
30 art, and are also within the scope of the invention.

In addition, possible variant forms of the VEGF-B
polypeptide which may result from alternative splicing, as are
known to occur with VEGF and VEGF-B, and naturally-occurring
allelic variants of the nucleic acid sequence encoding VEGF-B are

encompassed within the scope of the invention. Allelic variants
are well known in the art, and represent alternative forms or a
nucleic acid sequence which comprise substitution, deletion or
addition of one or more nucleotides, but which do not result in
5 any substantial functional alteration of the encoded polypeptide.

Such variant forms of VEGF-B can be prepared by targeting
non-essential regions of the VEGF-B polypeptide for modification.
These non-essential regions are expected to fall outside the
strongly-conserved regions of the VEGF/PDGF family of growth
10 factors. In particular, the growth factors of the VEGF family,
including VEGF-B, are dimeric, and VEGF, VEGF-B, VEGF-C, VEGF-D,
PlGF, PDGF-A and PDGF-B show complete conservation of eight
cysteine residues in the N-terminal domains, i.e. the PDGF/VEGF-
homology domains (Olofsson et al., Proc. Natl. Acad. Sci. USA,
15 1996 93 2576-2581; Joukov et al., EMBO J., 1996 15 290-298).
These cysteines are thought to be involved in intra- and inter-
molecular disulfide bonding. In addition there are further
strongly, but not completely, conserved cysteine residues in the
C-terminal domains. Loops 1, 2 and 3 of each subunit, which are
20 formed by intra-molecular disulfide bonding, are involved in
binding to the receptors for the PDGF/VEGF family of growth
factors (Andersson et al., Growth Factors, 1995 12 159-164).

Persons skilled in the art thus are well aware that in most
cases these cysteine residues should be preserved in any proposed
25 variant form, although there may be exceptions since receptor-
binding VEGF-B analogs are known in which one or more of the
cysteines is not conserved. Similarly, a skilled worker would
be aware that the active sites present in loops 1, 2, and 3 also
should be preserved. However, other regions of the molecule can
30 be expected to be of lesser importance for biological function,
and therefore offer suitable targets for modification. Modified
polypeptides can readily be tested for their ability to show the
biological activity of VEGF-B by routine activity assay
procedures such as the endothelial cell proliferation assay.

Preferably where amino acid substitution is used, the substitution is conservative, i.e. an amino acid is replaced by one of similar size and with similar charge properties. As used herein, the term "conservative substitution" denotes the

5 replacement of an amino acid residue by another, biologically similar residue, i.e., one that has similar properties. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine,

10 norleucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. Neutral hydrophilic amino acids which can be substituted for one another include asparagine, glutamine,

15 serine and threonine. The term "conservative substitution" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid.   Exemplary conservative substitutions are set out in the following Table A:

Table A

20   Conservative Substitutions I

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Aliphatic	
Non-polar	G A P
	I L V
Polar - uncharged	C S T M
	N Q
Polar - charged	D E
	K R
Aromatic	H F W Y
Other	N Q D E

Alternatively, conservative amino acids can be grouped as

35 described in Lehninger, [Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77] as set out in the following Table B.

Table B

Conservative Substitutions II

SIDE CHAIN

CHARACTERISTIC	AMINO ACID
Non-polar (hydrophobic)	
A. Aliphatic:	A L I V P
B. Aromatic:	F W
C. Sulfur-containing:	M
D. Borderline:	G
Uncharged-polar	
A. Hydroxyl:	S T Y
B. Amides:	N Q
C. Sulfhydryl:	C
D. Borderline:	G
Positively Charged (Basic):	K R H
Negatively Charged (Acidic):	D E

Exemplary conservative substitutions also are set out in the following Table C.

Table C

Conservative Substitutions III

	Original Residue	Exemplary Substitution
5	Ala (A)	Val, Leu, Ile
	Arg (R)	Lys, Gln, Asn
	Asn (N)	Gln, His, Lys, Arg
	Asp (D)	Glu
	Cys (C)	Ser
10	Gln (Q)	Asn
	Glu (E)	Asp
	His (H)	Asn, Gln, Lys, Arg
	Ile (I)	Leu, Val, Met, Ala, Phe,
	Leu (L)	Ile, Val, Met, Ala, Phe
15	Lys (K)	Arg, Gln, Asn
	Met (M)	Leu, Phe, Ile
	Phe (F)	Leu, Val, Ile, Ala
	Pro (P)	Gly
	Ser (S)	Thr
20	Thr (T)	Ser
	Trp (W)	Tyr, Phe
	Tyr (Y)	Trp, Phe, Thr, Ser
	Val (V)	Ile, Leu, Met, Phe, Ala

25   If desired, the VEGF-B proteins of the invention can be
modified, for instance, by amidation, carboxylation, or
phosphorylation, or by the creation of acid addition salts,
amides, esters, in particular C-terminal esters, and N-acyl
derivatives of the peptides of the invention. The proteins also

can be modified to create peptide derivatives by forming covalent or noncovalent complexes with other moieties. Covalently-bound complexes can be prepared by linking the chemical moieties to functional groups on the side chains of amino acids comprising

5 the peptides, or at the N- or C-terminus.

In particular, it is anticipated that the VEGF-B proteins can be conjugated to a reporter group, including, but not limited to a radiolabel, a fluorescent label, an enzyme (e.g., that catalyzes a colorimetric or fluorometric reaction), a substrate,

10 a solid matrix, or a carrier (e.g., biotin or avidin). T h e polypeptide can be linked to an epitope tag, such as the FLAG® octapeptide (Sigma, St. Louis, MO) or histidine, to assist in affinity purification. Also the polypeptides according to the invention may be labeled with a detectable label. The

15 polypeptide may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels may be used. Examples of radioactive labels include a radioactive atom or group, such as

20 ^{125}I or ^{32}P. Examples of non-radioactive labels include enzymatic labels, such as horseradish peroxidase or fluorimetric labels, such as fluorescein-5-isothiocyanate (FITC). Labeling may be direct or indirect, covalent or non-covalent.

The modified polypeptides can readily be tested for their

25 ability to show the biological activity of VEGF-B by routine activity assay procedures such as the fibroblast proliferation assay.

It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means

30 or by recombinant means, or may be purified from natural sources.

As used herein, the term "comprising" means "included but not limited to". The corresponding meaning applies to the word "comprises".

A third aspect of the invention provides vectors comprising
the nucleic acid molecule of the first aspect of the invention,
and host cells transformed or transfected with nucleic acids
molecules or vectors of the invention. These may be eukaryotic
5 or prokaryotic in origin. These cells are particularly suitable
for expression of the polypeptide of the invention, and include
insect cells such as Sf9 or HF cells, obtainable from the
American Type Culture Collection, infected with a recombinant
baculovirus, and the human embryo kidney cell line 293-EBNA
10 transfected by a suitable expression plasmid. Preferred vectors
of the invention are expression vectors in which a nucleic acid
according to the invention is operatively connected to one or
more appropriate promoters and/or other control sequences, 'such
that appropriate host cells transformed or transfected with the
15 vectors are capable of expressing the polypeptide of the
invention. Other preferred vectors are those suitable for
transfection of mammalian cells, or for gene therapy, such as
adenoviral-, vaccinia- or retroviral-based vectors or liposomes.
A variety of such vectors are known in the art.

20   The invention also provides a method of making a vector
capable of expressing a polypeptide encoded by a nucleic acid
according to the invention, comprising the steps of operatively
connecting the nucleic acid molecule of the first aspect to one
or more appropriate promoters and/or other control sequences, as
25 described above.

        The invention further provides a method of making a
polypeptide according to the invention, comprising the steps of
expressing a nucleic acid or vector of the invention in a
host cell, and isolating the polypeptide from the host cell or
30 from the host cell's growth medium.

        The polypeptide according to the invention may be employed
in combination with a suitable pharmaceutical carrier. The
resulting compositions comprise an effective amount of

glycosylated VEGF-B or a pharmaceutically acceptable non-toxic
salt thereof, and a pharmaceutically acceptable solid or liquid
carrier or adjuvant. Examples of such a carrier or adjuvant
include, but are not limited to, saline, buffered saline,
5 Ringer's solution, mineral oil, talc, corn starch, gelatin,
lactose, sucrose, microcrystalline cellulose, kaolin, mannitol,
dicalcium phosphate, sodium chloride, alginic acid, dextrose,
water, glycerol, ethanol, thickeners, stabilizers, suspending
agents and combinations thereof. Such compositions may be in the
10 form of solutions, suspensions, tablets, capsules, creams,
salves, elixirs, syrups, wafers, ointments or other conventional
forms. The formulation to suit the mode of administration.
Compositions can comprise a glycosylated VEGF-B and optionally
further comprise one or more of PDGF-A, PDGF-B, VEGF, non-
15 glycosylated VEGF-B, VEGF-C, VEGF-D, PlGF and/or heparin.
Compositions comprising the glycosylated VEGF-B will contain from
about 0.1% to 90% by weight of the active compound(s), and most
generally from about 10% to 30%.

For intramuscular preparations, a sterile formulation,
20 preferably a suitable soluble salt form of the glycosylated VEGF-
B, such as hydrochloride salt, can be dissolved and administered
in a pharmaceutical diluent such as pyrogen-free water
(distilled), physiological saline or 5% glucose solution. A
suitable insoluble form of the compound may be prepared and
25 administered as a suspension in an aqueous base or a
pharmaceutically acceptable oil base, e.g. an ester of a long
chain fatty acid such as ethyl oleate.

In a further aspect, the invention provides a method for
making a soluble VEGF-B$_{167}$ from a host cell and a method for
30 increasing an amount of a soluble VEGF-B$_{167}$, VEGF-B$_{186}$ or VEGF-B$_{Ex1-5}$
protein from a host cell. These methods comprise inserting at
least one putative N-glycosylation site into a nucleotide
sequence which codes for VEGF-B$_{167}$, VEGF-B$_{186}$ or VEGF-B$_{Ex1-5}$ protein;

transforming or transfecting said nucleotide sequence with the
inserted N-glycosylation site into a host cell; culturing the
transfected host cell in a growth medium such that said
nucleotide sequence with inserted N-glycosylation site is
5 expressed; and isolating the expressed polypeptide from the
growth medium in which said host cell was cultured. These
methods can further comprise exposing the cultured transfected
host cell to heparin after said polypeptide is expressed.

10 BRIEF DESCRIPTION OF THE DRAWINGS
        The invention will be described in further detail
hereinafter with reference to the accompanying drawings in which:
        Fig. 1 is an alignment of the amino acid sequences of the
VEGF homology domain (VHD) of VEGF-A and PlGF with VEGF-B;
15   Fig. 2 is a diagram of plasmid pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT
containing a nucleotide sequence encoding VEGF-B$_{186}$ having an
N-glycosylation site incorporated therein;
        Fig. 3 is a diagram of plasmid pSecTagA-hVEGF-B$_{167}$-H$_6$-NXT
containing a nucleotide sequence encoding VEGF-B$_{167}$ having an
20 N-glycosylation site incorporated therein;
        Fig. 4 is a diagram of plasmid pSecTagA-hVEGF-B-Exon1-5-H$_6$-
NXT containing a nucleotide sequence encoding exons 1-5 of VEGF-B
having an N-glycosylation site incorporated therein;
        Fig. 5 shows the expression of hVEGF-B$_{167}$ with and without
25 the potential glycosylation site (NXT);
        Fig. 6 shows the expression of hVEGF-B$_{167}$ and hVEGF-B$_{186}$ with
and without the potential glycosylation site (NXT);
        Fig. 7 shows the expression and receptor binding of hVEGF-
B$_{167}$ and hVEGF-B$_{186}$ with and without the potential glycosylation
30 site (NXT); and
        Fig. 8 shows the expression and receptor binding of
polypeptide encoded by exons 1-5 of hVEGF-B with and without the
potential glycosylation site (NXT).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1: Introduction of the glycosylation site

5   As mentioned before, VEGF-B is a PDGF/VEGF family member
that is completely devoid of any N-glycosylation. To analyze the
effects of N-glycosylation on VEGF-B, a N-glycosylation site was
introduced into VEGF-B. To determine the most appropriate site
to introduce a mutation that would lead to N-glycosylation of
10 VEGF-B, the amino acid sequences of the first 99 amino acids of
VEGF-A, PlGF and VEGF-B, respectively, were aligned (see Fig. 1).
The N-glycosylation sites of VEGF-A and PlGF at amino acids 65-67
are italicized in Fig. 1. Nucleotides encoding a putative N-
glycosylation site (NXT) were inserted at the position
15 corresponding to nucleotides 286-294 of hVEGF-B (SEQ ID NO:1).
The replaced nucleotides normally found at positions 286-294
encode the amino acid residues QVR and these amino acid residues
are bolded in Fig. 1.

20 Example 2: Preparation of Recombinant Vectors
       Six mammalian expression vectors for both naturally
occurring isoforms of VEGF-B (i.e., VEGF-B$_{167}$ and VEGF-B$_{186}$) and
for an artificial splice variant (comprising exons 1 to 5 only)
were constructed with and without the putative N-glycosylation
25 site.
       Using PCR, nucleotides coding for a histidine tag were added
to the C-terminal end of a nucleotide sequence coding for hVEGF-
B$_{186}$. A nucleotide sequence coding for hVEGF-B$_{186}$-H$_6$ was then
inserted into pSecTagA (Invitrogen, Carlsbad, California) using
30 standard cloning procedures to construct pSecTagA-hVEGF-B$_{186}$-H$_6$.
The full sequence of pSecTagA-hVEGF-B$_{186}$-H$_6$ is given in SEQ ID
NO:7.

To construct pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT, a PCR product of
covering nucleotides 1-325 from Genebank Acc. No. U48801 was
produced which introduced a N-glycosylation site at nucleotide
positions   289-297   using   the   3'primer: 5'-
5 TCGGTACCGGATCATGAGGATCTGCATGGTGACGTTGTGCTGCCCAGTGGCCA-3' (SEQ ID
NO:8).   This PCR product was then cloned into a plasmid with
full-length hVEGF-B$_{186}$ where it used to replace the corresponding
sequence to produce hVEGF-B$_{186}$-NXT.   A histidine tag was then
added by cloning together the N-terminal portion of hVEGF-B$_{186}$-NXT
10 with .the C-terminal portion of hVEGF-B$_{186}$-H$_6$ using standard
cloning procedures to produce hVEGF-B$_{186}$-H$_6$-NXT.   The nucleotide
sequence coding for hVEGF-B$_{186}$-H$_6$-NXT was then inserted into
pSecTagA (Invitrogen) using standard cloning procedures to
construct pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT.   The full sequence of
15 pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT is given in SEQ ID NO:9, and the
plasmid is illustrated in Fig. 2.

To construct pSecTagA-hVEGF-B$_{167}$-H$_6$, a 349 bp PCR product was
produced covering nucleotides 250-567 from Genebank Acc. No.
U48801, nucleotides coding for the histidine tag, a stop codon,
20 the NotI restriction site and terminal clamp nucleotides using
the 5' primer: 5'-CCTGACGATGGCCTGGAGTGT-3' (SEQ ID NO:10) and the
3'   primer: 5'-GAGCGGCCGCTCAATGATGATGATGATGAT
GCCTTCGCAGCTTCCGGCAC-3' (SEQ ID NO:11) and hVEGF-B$_{167}$ as the
template. The 349 bp PCR product was cut with KpnI and NotI and
25 the KpnI-NotI fragment was inserted into pSecTagA-hVEGF-B$_{186}$-H$_6$ to
replace the KpnI-NotI fragment removed from this vector using
standard cloning procedures.   The full sequence of pSecTagA-
hVEGF-B$_{167}$-H$_6$ is given in SEQ ID NO:12.

Similarly, pSecTagA-hVEGF-B$_{167}$-H$_6$-NXT was constructed as
30 above except the KpnI-NotI fragment was inserted into pSecTagA-
hVEGF-B$_{186}$-H$_6$-NXT to replace the KpnI-NotI fragment removed from
this vector. The full sequence of pSecTagA-hVEGF-B$_{167}$-H$_6$-NXT is
given in SEQ ID NO:13, and the plasmid is illustrated in Fig. 3.

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To construct pSecTagA-hVEGF-B$_{Ex1-5}$-H$_6$, a 443 bp PCR product was obtained covering nucleotides 1-411 from Genebank Acc. No. U48801, nucleotides coding for the histidine tag, a stop codon, the *NotI* restriction site and terminal clamp nucleotides using
5 the 5' primer: 5'-CACCATGAGCCCTCTGCTCC-3' (SEQ ID NO:14) and 3' primer: 5'-GAGCGGCCGCTCAGTGGTGATGATGATGGTCTGGCTTCACAGCACTG-3' (SEQ ID NO:15) and hVEGF-B$_{167}$ as the template. The PCR product was cut with *KpnI* and *NotI* and the resulting 320 bp fragment was inserted into pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT to replace the *KpnI*-*NotI*
10 removed from this vector using standard cloning procedures. The full sequence of pSecTagA-hVEGF-B$_{Ex1-5}$-H$_6$ is given in SEQ ID NO:16.

To construct pSecTagA-hVEGF-B$_{Ex1-5}$-H$_6$-NXT, the same procedures as above were used except the *KpnI*-*NotI* fragment was inserted into pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT to replace the *KpnI*-*NotI* fragment
15 removed from this vector. The full sequence of pSecTagA-hVEGF-B$_{Ex1-5}$-H$_6$-NXT is given in SEQ ID NO:17, and the plasmid is illustrated in Fig. 4.

The following Table D lists the expression vectors for the naturally occurring 186 and 167 amino acid isoforms of VEGF-B and
20 for the artificial splice variant (comprising exon 1 to 5 only), constructed with and without the potential glycosylation site (NXT).

Table D

Construct Name	Protein
pSecTagA-hVEGF-B$_{186}$-H$_6$	histidine-tagged VEGF-B$_{186}$
pSecTagA-hVEGF-B$_{186}$-H$_6$-NXT	histidine-tagged and N-glycosylated VEGF-B$_{186}$
pSecTagA-hVEGF-B$_{167}$-H$_6$	histidine-tagged VEGF-B$_{167}$
pSecTagA-hVEGF-B$_{167}$-H$_6$-NXT	histidine-tagged and N-glycosylated VEGF-B$_{167}$
pSecTagA-hVEGF-B-Exon1-5-H$_6$	histidine-tagged VEGF-B Exons 1 to 5 only
pSecTagA-hVEGF-B-Exon1-5-H$_6$-NXT	histidine-tagged and N-glycosylated VEGF-B Exons 1 to 5 only

Example 3: Transfection and Expression of Recombinant Proteins

The six mammalian expression vectors of human VEGF-B described above along with expression vectors containing histidine-tagged VEGF (positive control), a histidine-tagged VHD of VEGF-C (negative control) and histidine-tagged hybrid 84-11 (positive control), respectively, were transfected into 293T cells using CaPO$_4$-mediated transfection according to procedures described in Sambrook, J. et al., Molecular Cloning, A Laboratory Manual, (Cold Spring Harbor Press, Cold Spring Harbor, NY), 16.33-16.36 (1989). 48 hours after transfection, the cells were metabolically labeled with S^{35} methionine and S^{35} cysteine (Promix, Amersham) for 12 to 24 hours. The conditioned supernatant was subjected to immunoprecipitation with an

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antiserum specific to human VEGF-B (874) and a monoclonal antibody specific to pentahistidine (H_5 mAb, Qiagen).

As seen in Figs. 5 through 8, the three constructs produced with the inserted putative N-glycosylation site are glycosylated.

5   As can be seen from Figs. 5-7, comparison of supernatants and lysates and using heparin to release cell bound proteins shows that VEGF-B_{167} is almost completely retained at the cell surface or within the cell. About a 50 fold increase of VEGF-B_{167} can be detected in the supernatant upon glycosylation (Fig. 5).

10 As shown in Figs. 6 and 7, VEGF-B_{167} is released into the supernatant by treatment with 100 μg/ml heparin two hours prior to harvest. It was also found that approximately two times more glycosylated VEGF-B_{167} can be detected in the supernatant of non-heparin treated 293T cells as compared to non-glycosylated VEGF-

15 B_{167} treated with 200 μg/ml heparin for two hours prior to harvesting. In addition, there is about a three fold increase in the amount of the glycosylated VEGF-B_{167} detected in the supernatant by treatment with 200 μg/ml heparin two hours prior to harvest as compared to glycosylated VEGF-B_{167} without heparin

20 treatment, and approximately a six fold increase in the amount of the glycosylated VEGF-B_{167} detected in the supernatant by treatment with 200 μg/ml heparin two hours prior to harvest as compared to the amount of non-glycosylated VEGF-B_{167} detected in the supernatant with the same heparin treatment.

25   Figs. 6 and 7 show that VEGF-B_{186} is also partially retained at the cell surface and within the cell. In contrast to VEGF-B_{167}, almost all of the VEGF-B_{186} is released into the supernatant upon glycosylation and heparin treatment (Figs. 6 and 7). There seems to be no significant difference in the amount of VEGF-B_{186}

30 found in the supernatant between heparin-treated and untreated 293T cells. Thus the difference of VEGF-B_{186} and N-glycosylated VEGF-B_{186} protein levels in the supernatant (approximately three times more glycosylated VEGF-B_{186}) seems to be mainly due to

enhanced secretion and/or production and not due to the release
of cell surface bound protein.

Fig. 8 shows that VEGF-B$_{Exon1-5}$ is only efficiently released
into the medium if it is N-glycosylated (over a 50 fold increase
5 in soluble protein). This is unexpected since the signals
retaining VEGF-B at the cell surface are thought to reside in the
exon 6 and 7 encoded domains (Fig. 8). Treatment with heparin
was not determined for this same reason.

10 Example 4: VEGF receptor 1 binding of recombinant proteins
The ability of the recombinant VEGF-B to bind VEGF receptor
1 (VEGFR-1) was analyzed using soluble fusion proteins consisting
of the extracellular domain of VEGFR-1 and the Fc portion of
human IgG1 (VEGFR-1-Fc). Biosynthetically labeled conditioned
15 medium derived from 293T cells transfected as above in Example
3 were immunoprecipitated with protein A sepharose (PAS) bound
to the VEGFR-1-Ig. Beads were washed three times with PBS, the
bound protein eluted and resolved by reducing SDS-PAGE (15%).
The dried gels were exposed to phosphoimager plates for 12-24
20 hours. Additionally, the cell lysates were immunoprecipitated
with H$_5$ mAb.

When significant amounts of VEGF-B were present in the
supernatant, binding to VEGFR-1 could be observed. This was seen
with VEGF-B$_{186}$-H$_6$ after treatment with 100 μg/ml heparin two hours
25 prior to harvest, VEGF-B$_{186}$-NXT-H$_6$ and VEGF-B Exon 1-5-NXT-H$_6$
(Figs. 7 and 8).

Example 5: Stimulation of BaF3 VEGFR-01EC/EpoR Cell Survival
The effects of introducing the N-glycosylation site into
30 VEGF-B can be assayed by measuring the ability of conditioned
media from cells transfected with VEGF-B167 and VEGF-B167-NXT
and/or VEGF-B186 and VEGF-B186-NXT to stimulate the survival of
BaF3 VEGFR-01EC/EpoR cells. For the assay, BaF3 cells are used

that are stably transfectd with a chimeric receptor consisting
of the extracellular domain of VEGF receptor 1 and the
intracellular domain of the erythropoietin receptor. For
survival, these cells need either interlukin-3 or any growth
5 factor capable of binding VEGFR-1, e.g., VEGF-A, VEGF-B or PlGF.
Cells are plated to 96-well plates at a density of 20,000/well
and grown in the presence of different amounts of medium
conditioned by 293T cells that have been transfected with VEGF-
B167 and VEGF-B167-NXT, VEGF-B186 and VEGF-B186-NXT, or both.
10 Conditioned medium from 293T cells transfected with a mock (i.e.,
empty) vector may be used as a control. Prior to the assay, the
conditioned medium should be cleared from potentially interfering
proteins by immunoprecipitation using appropriate antibodies.
For example, VEGF-A may be cleared from the conditioned medium
15 prior to the assay using a mixture of monoclonal and polyclonal
anti-hVEGF antibodies, commercially available from R&D Systems,
Minneapolis, Minnesota. It is not necessary to preclear medium
of PlGF as, the amounts expressed by COS cells (if any) are
negligible and its effects are not visible in the baseline noise.
20 After 48 hours, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-
diphenyl-tetrazolium bromide thiazole blue) colorimetric assay
may be performed and data collected at 540 nm using a
microtitreplate reader.

        To create the BglII site in the coding sequence of human
25 VEGFR-1 just before the transmembrane domain, basepairs 1998-2268
of VEGFR-1 were PCR amplified with primers 5'-
CCTCAGTGATCACACAGTGG-3', containing the endogenous BclI site, and
5'-CAGAGATCTATTAGACTTGTCC-3', containing a BglII site, and the
PCR fragment was cloned into the BclI-BglII sites of VEGFR-1 in
30 pBlueScript SKII+ (Stratagene) vector. The transmembrane and
intracellular domains of the human erythropoietin receptor were
excised from EpoR x B+B/pcDNAI and subcloned into the resulting
plasmid as a BglII/NotI fragment. The EpoR x B+B is a clone of

EpoR which has an internal BglII site added at the putative
transmembrane (TM)/extracellular (EC) domain junction for the in-
frame ligation of RTK extracellular domains. The vector backbone
is pCDNA1-amp (~5.4 kb, the original 1.75 kb EpoR clone was
5 subcloned into pCDNA1-amp using KpnI, the sequence was reported
by the Lodish Laboratory, MIT). An ~1 kb fragment can be excised
from this clone using BglII (5')- NotI (3') digest which contains
the TM and cytoplasmic domain of EpoR.

The VEGFR-1/EpoR construct was finally subcloned into the
10 pEF-BOS vector (Mizushima et al., Nucleic Acids Research,
18(17):5322 Sept. 11, 1990) as a KpnI/NotI fragment. The
resulting plasmid was electroporated into BaF3 cells and stable
cell pools were generated by selection with 250 micrograms/mL
zoecin.

15 The foregoing description and examples have been set forth
merely to illustrate the invention and are not intended to be
limiting. Since modifications of the disclosed embodiments
incorporating the spirit and substance of the invention may occur
to persons skilled in the art, the invention should be construed
20 broadly to include everything within the scope of the appended
claims and equivalents thereof.

WHAT IS CLAIMED IS:

1.   An isolated nucleic acid molecule comprising:
     a polynucleotide sequence selected from the group
consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a
polynucleotide sequence which hybridizes under stringent
conditions with at least one of the foregoing sequences;
and
     a nucleotide sequence encoding at least one putative
N-glycosylation site inserted therein.

2.   An isolated polypeptide having an amino acid sequence
selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4
and SEQ ID NO:6 and having at least one putative N-glycosylation
site inserted therein.

3.   The isolated nucleic acid molecule of claim 1, wherein
the at least one putative N-glycosylation site consists of a
nucleotide sequence that encodes an amino acid sequence of NXT.

4.   The isolated nucleic acid molecule of claim 1, wherein
the at least one putative N-glycosylation site is inserted at
nucleotides 286-294 of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

5.   An isolated polypeptide produced by expression of the
nucleic acid molecule of claim 1.

6.   An isolated polypeptide of claim 2 which binds a
Vascular Endothelial Growth Factor Receptor-1.

7.   A vector comprising a nucleic acid molecule of claim
1.

8. A host cell transformed or transfected with a vector according to claim 7.

9. A pharmaceutical composition comprising an effective amount of a polypeptide of claim 2.

10. The pharmaceutical composition of claim 9, further comprising heparin.

11. A method of making a soluble VEGF-B$_{167}$ from a host cell, comprising:

inserting at least one putative N-glycosylation site into a nucleotide sequence of SEQ ID NO:1;

transforming or transfecting said nucleotide sequence with inserted N-glycosylation site into a host cell;

culturing the transfected host cell in a growth medium such that said nucleotide sequence with inserted N-glycosylation site is expressed; and

isolating the expressed polypeptide from the growth medium in which said host cell was cultured.

12. The method of claim 11, further comprising exposing the cultured transfected host cell to heparin after said polypeptide is expressed.

13. The method of claim 11, wherein the at least one putative N-glycosylation site consists of a nucleotide sequence that encodes an amino acid sequence of NXT.

14. The method of claim 11, wherein the nucleotide sequence encoding the at least one putative N-glycosylation site is inserted at nucleotides 286-294 of SEQ ID NO:1.

15. A method of increasing an amount of a soluble VEGF-B$_{167}$, VEGF-B$_{186}$ or VEGF-B$_{Ex1-5}$ polypeptide from a host cell, comprising:

inserting at least one putative N-glycosylation site into a nucleotide sequence selected from the group of nucleotides sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5;

transforming or transfecting said nucleotide sequence with inserted N-glycosylation site into a host cell;

culturing the transfected host cell in a growth medium such that said nucleotide sequence with inserted N-glycosylation site is expressed; and

isolating the expressed polypeptide from the growth medium in which said host cell was cultured.

16. The method of claim 15, further comprising exposing the cultured transfected host cell to heparin after said polypeptide is expressed.

17. The method of claim 15, wherein the at least one putative N-glycosylation site consists of a nucleotide sequence that encodes an amino acid sequence of NXT.

18. The method of claim 15, wherein the nucleotide sequence encoding the at least one putative N-glycosylation site is inserted at nucleotides 286-294 of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

Fig. 1

hVEGF-A	1	HHEVVKFMDV	YQRSYCHPIE	TLVDIFQEYP	DEIEYIFKPS	CVPLMRCGGC	50
hPIGF	1	EVEVVPFQEV	WGRSYCRALE	RLVDVVSEYP	SEVEHMFSPS	CVSLLRCTGC	50
hVEGF-B	1	QRKVVSWIDV	YTRATCQPRE	VVVPLTVELM	GTVAKQLVPS	CVTVQRCGGC	50
hVEGF-A	51	CNDEGLECVP	TEESNITMQI	MRIKPHQGQH	IGEMSFLQHN	KCECRPKK-D	99
hPIGF	51	CGDEDLHCVP	VETANVTMQL	LKIRSGDRPS	YVELTFSQHV	RCECRPLR-E	99
hVEFG-B	51	CPDDGLECVP	TGQHQVRMQI	LMIR-YPSSQ	LGEMSLEEHS	QCECRPKKKD	99

Fig. 2

Fig. 3

Fig. 4

Fig. 5
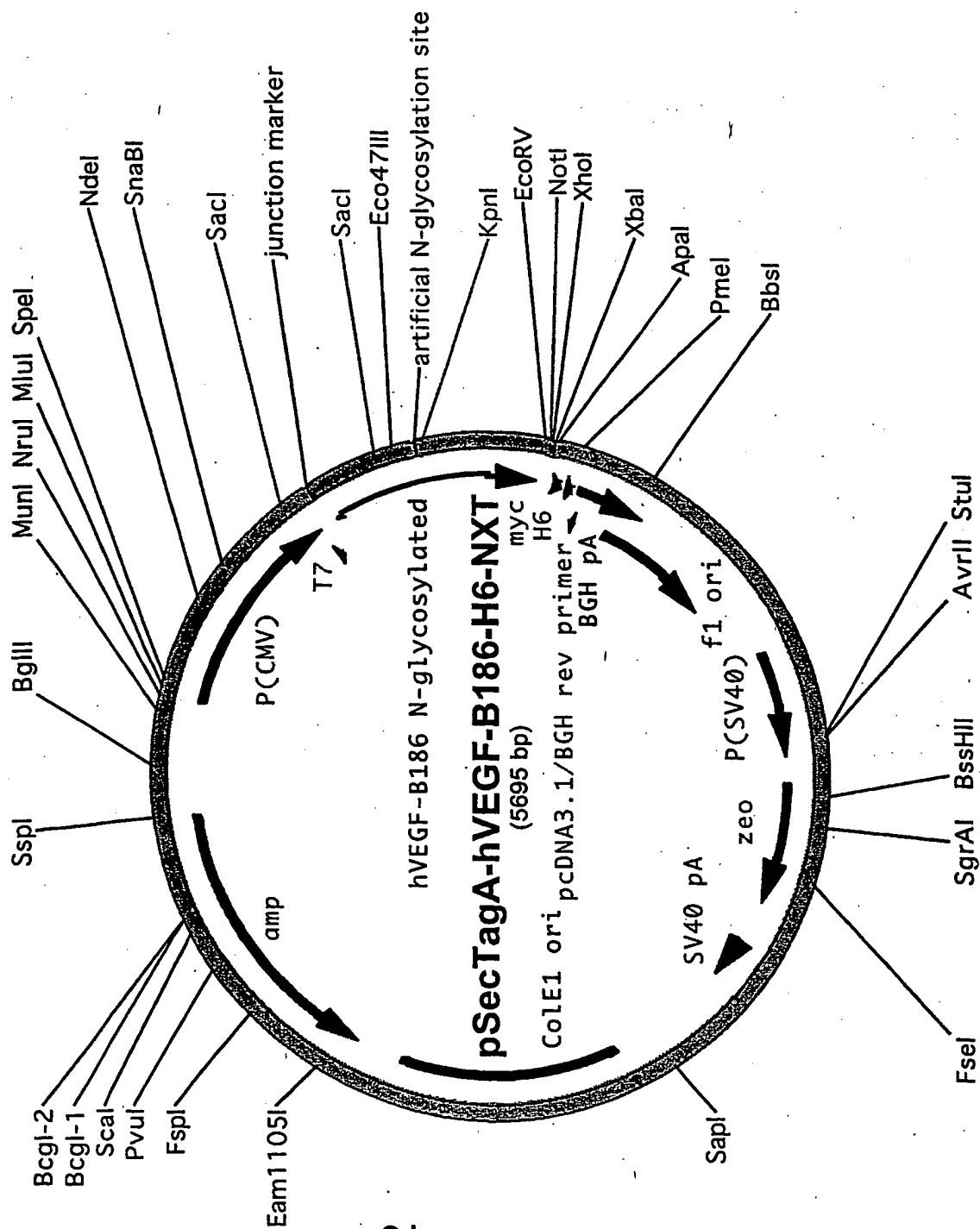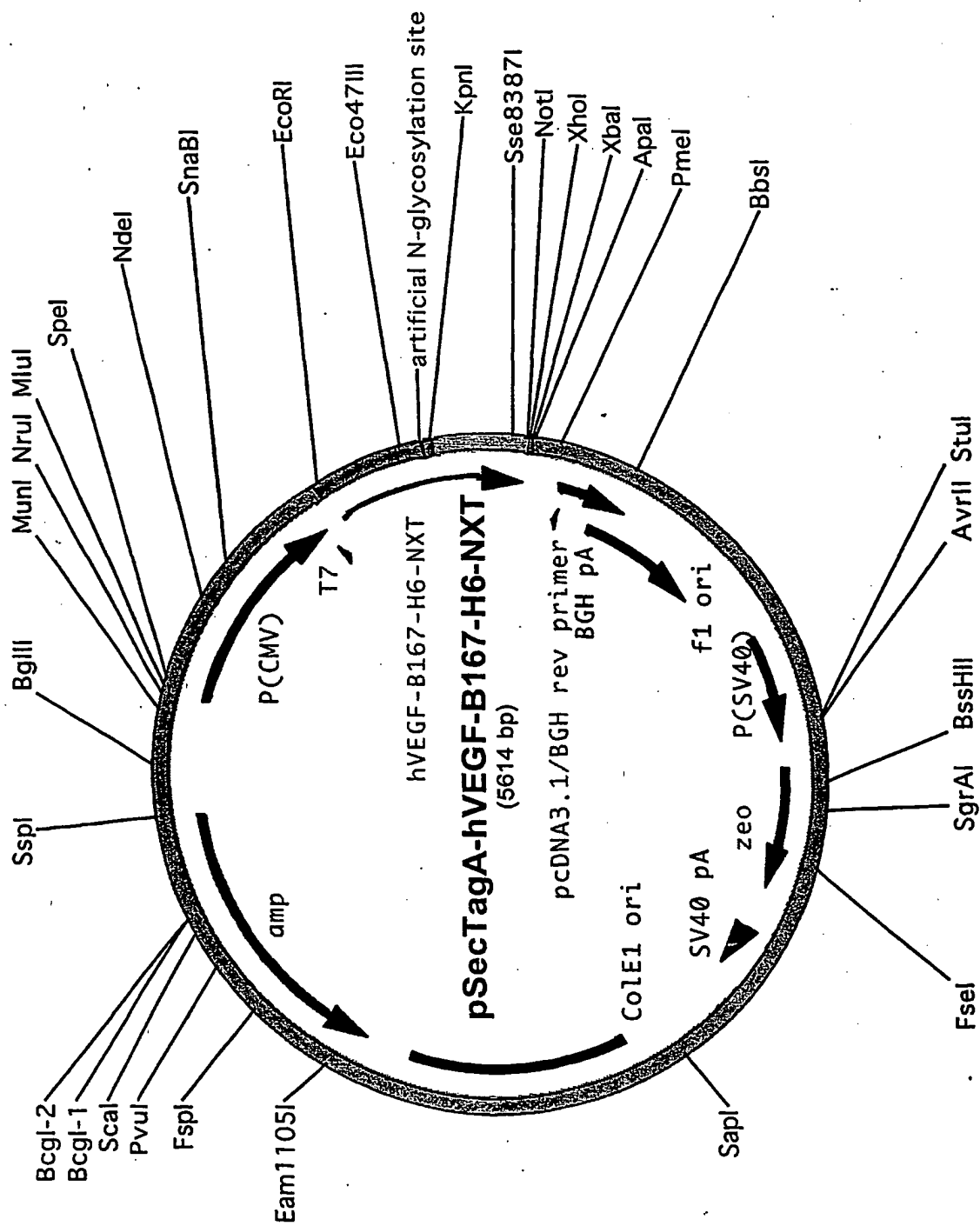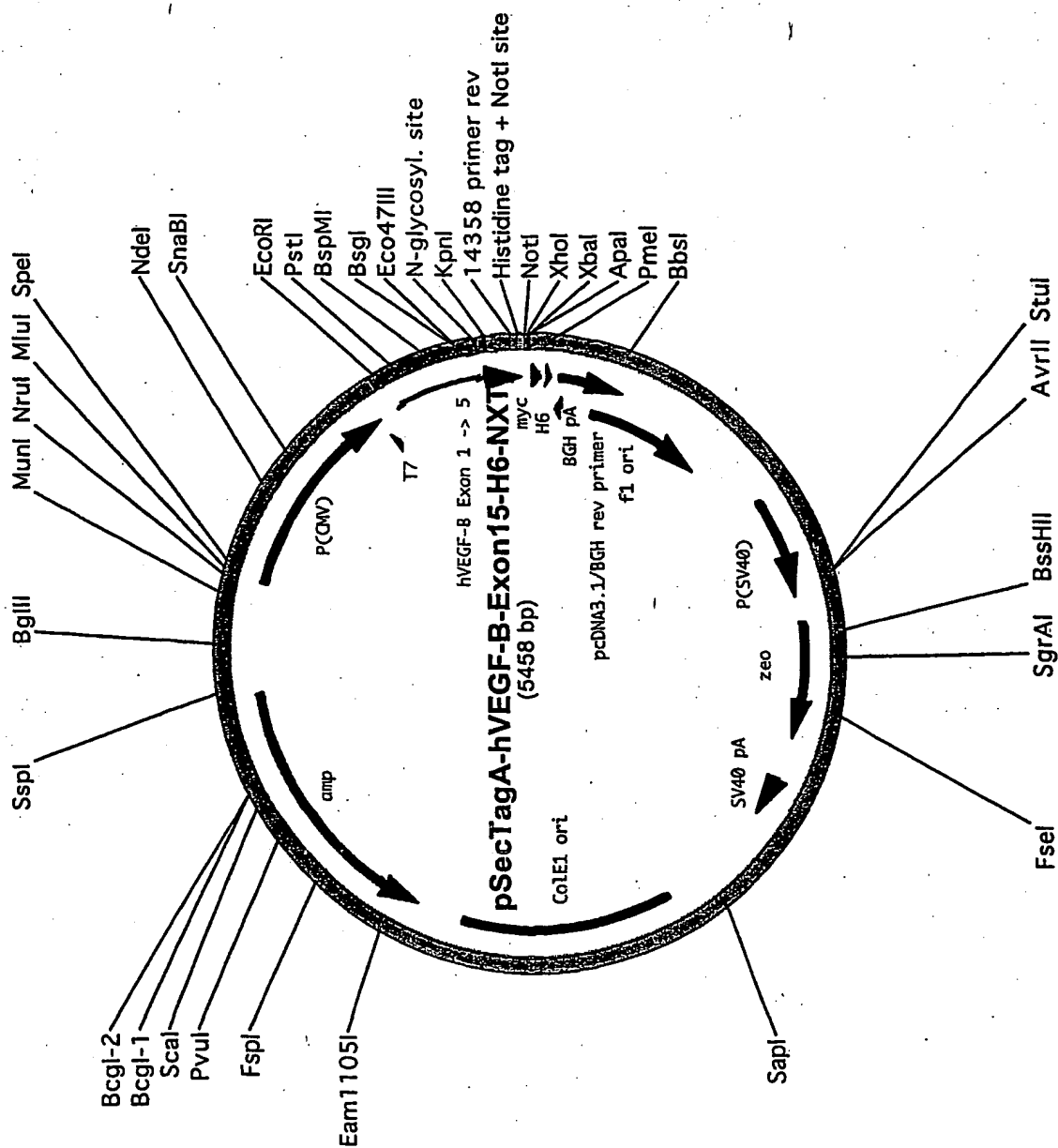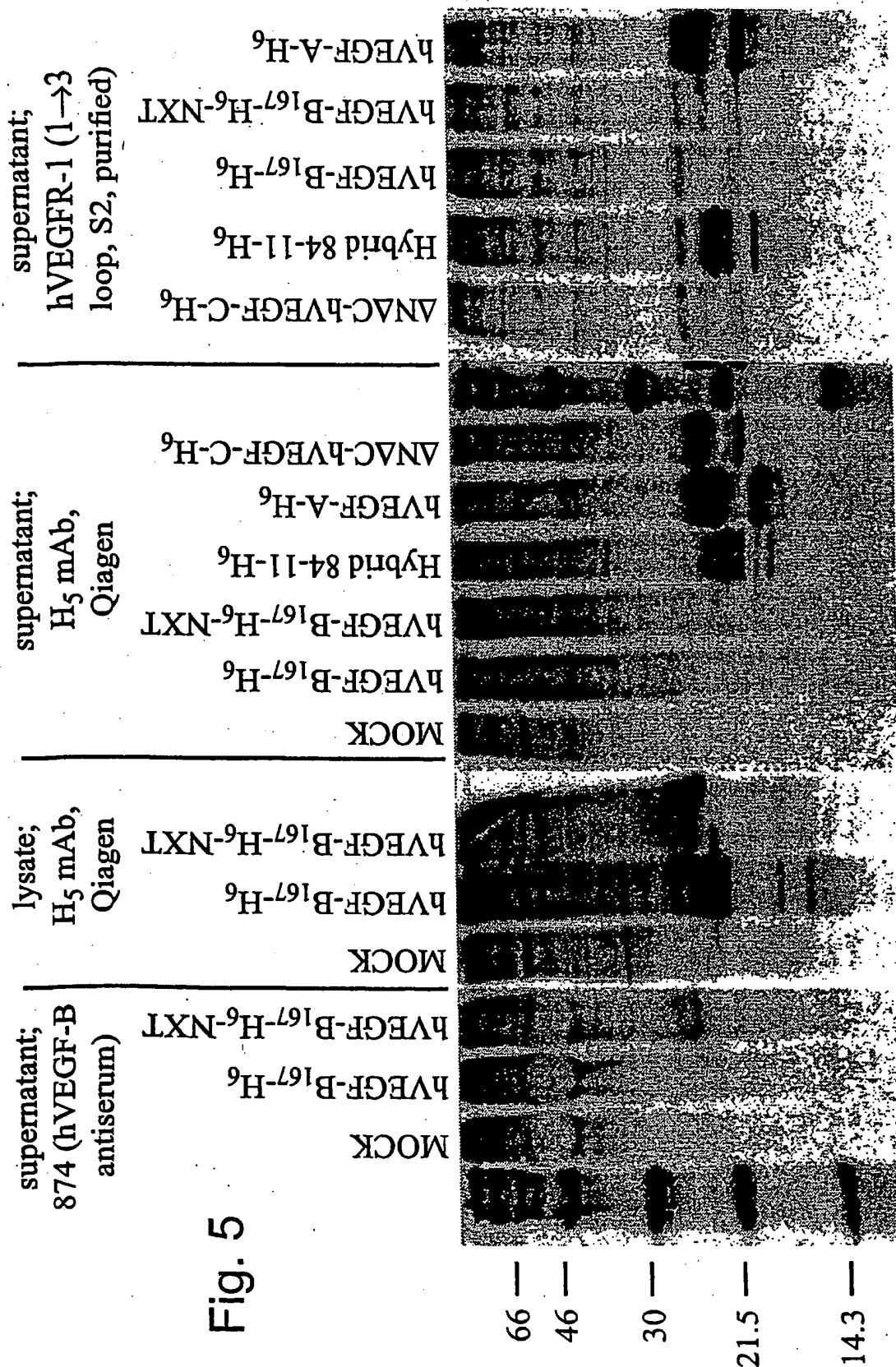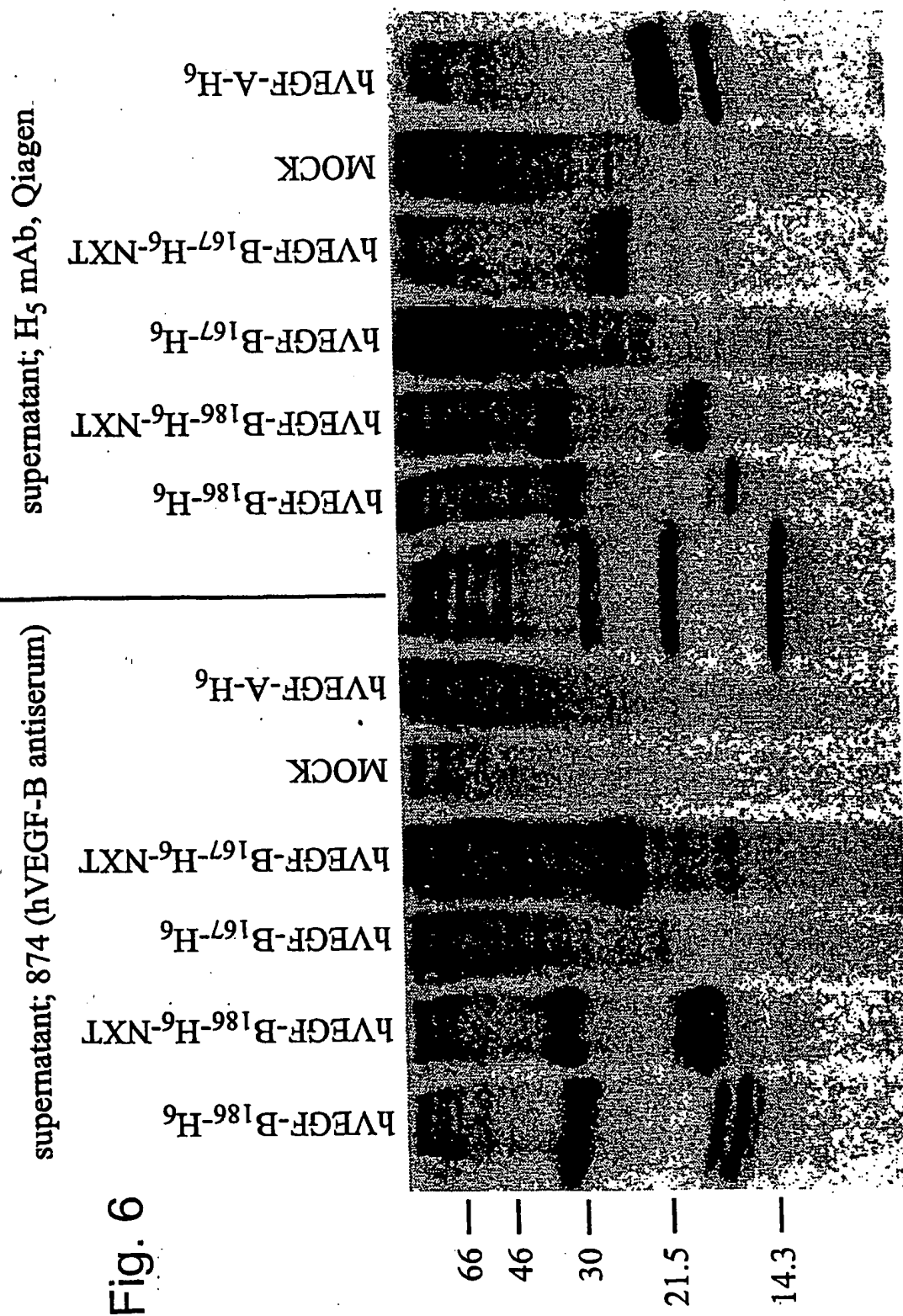
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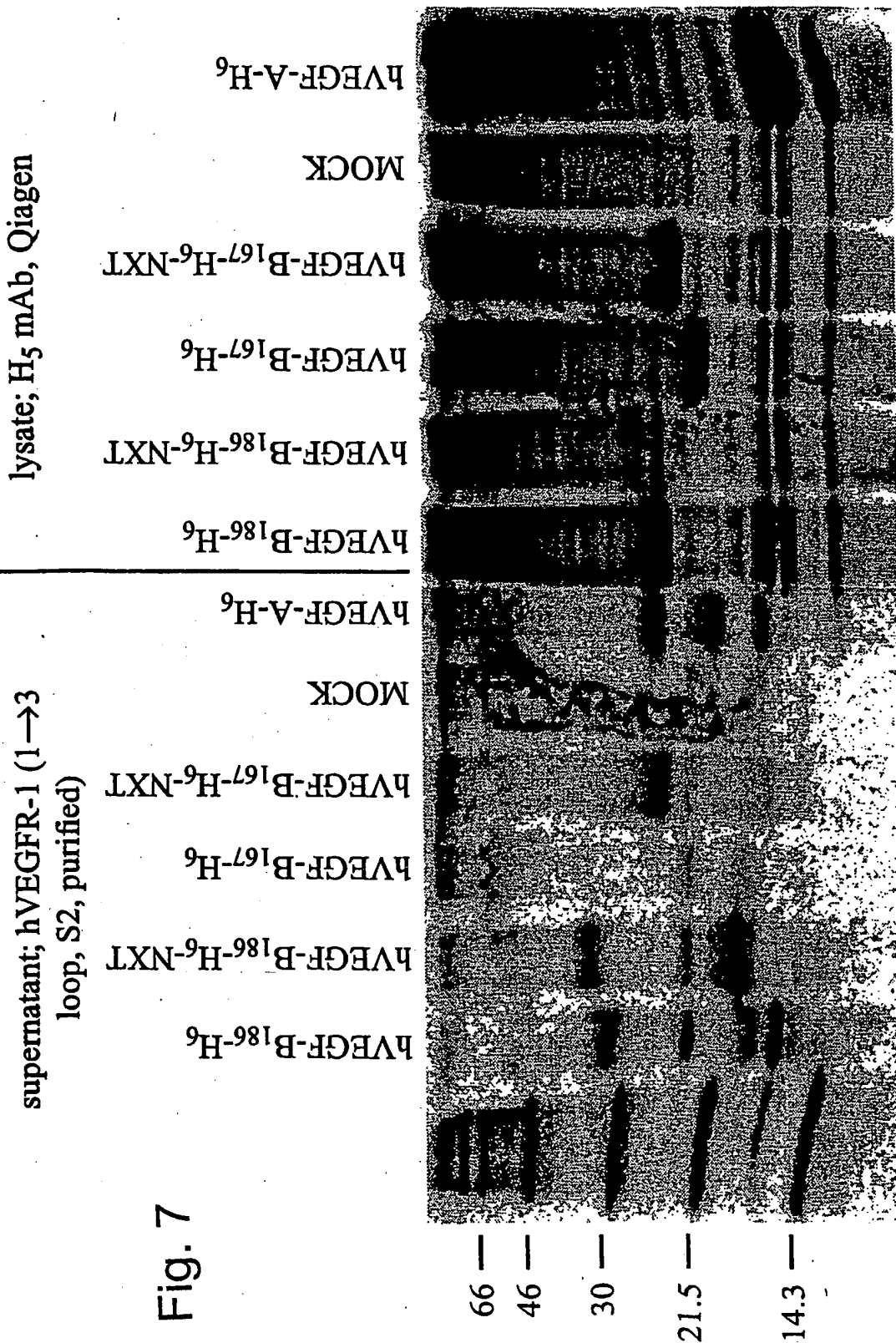
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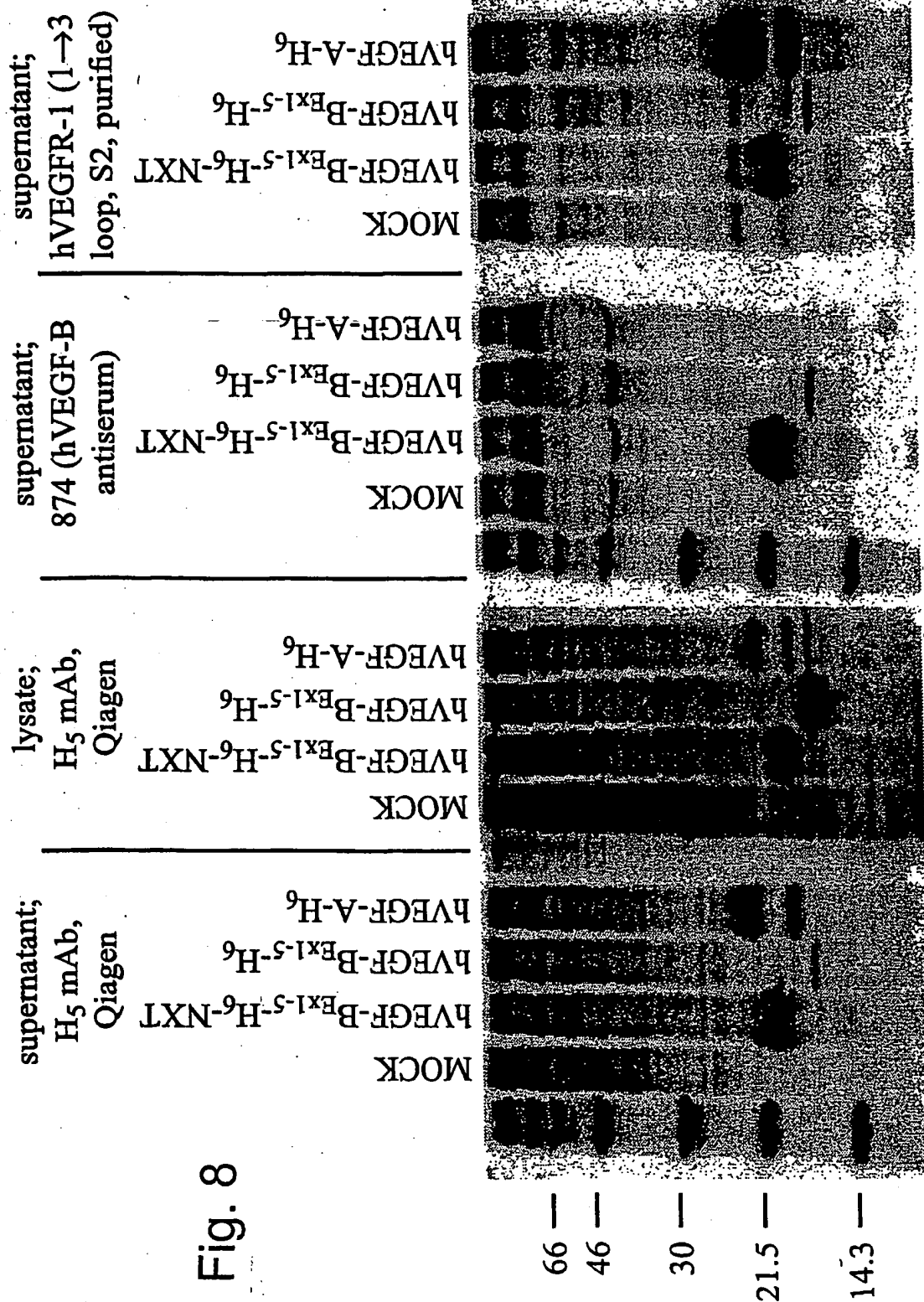
Fig. 8

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   ALITALO, Kari

   OLOFSSON, Birgitta

   ERIKSSON, Ulf

<120> GLYCOSYLATED VEGF-B AND METHOD FOR INCEREASING THE

   AMOUNT OF SOLUBLE VEGF-B

<130> 1064-48929PV Markku JELTSCH et al

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    -5   -1 1   5   10

agg aaa gtg gtg tca tgg ata gat gtg tat act cgc gct acc tgc cag   144
Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
            15   20   25

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Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val
        30   35   40

gcc aaa cag ctg gtg ccc agc tgc gtg act gtg cag cgc tgt ggt ggc   240
Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
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tgc tgc cct gac gat ggc ctg gag tgt gtg ccc act ggg cag cac caa   288
Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
60   65   70   75

gtc cgg atg cag atc ctc atg atc cgg tac ccg agc agt cag ctg ggg   336
Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly
            80   85   90

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aag gac agt gct gtg aag cca gac agc ccc agg ccc ctc tgc cca cgc    432
Lys Asp Ser Ala Val Lys Pro Asp Ser Pro Arg Pro Leu Cys Pro Arg
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Cys Thr Gln His His Gln Arg Pro Asp Pro Arg Thr Cys Arg Cys Arg
          125              130              135


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Cys Arg Arg Arg Ser Phe Leu Arg Cys Gln Gly Arg Gly Leu Glu Leu
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Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln
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agg aaa gtg gtg tca tgg ata gat gtg tat act cgc gct acc tgc cag   144
Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
                15   20   25

ccc cgg gag gtg gtg gtg ccc ttg act gtg gag ctc atg ggc acc gtg   192
Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val

            30   35   40

gcc aaa cag ctg gtg ccc agc tgc gtg act gtg cag cgc tgt ggt ggc   240
Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
    45   50   55

tgc tgc cct gac gat ggc ctg gag tgt gtg ccc act ggg cag cac caa   288
Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
60   65   70   75

gtc cgg atg cag atc ctc atg atc cgg tac ccg agc agt cag ctg ggg   336
Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly
              80   85   90

gag atg tcc ctg gaa gaa cac agc cag tgt gaa tgc aga cct aaa aaa   384
Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
            95   100   105

aag gac agt gct gtg aag cca gac agg gct gcc act ccc cac cac cgt   432
Lys Asp Ser Ala Val Lys Pro Asp Arg Ala Ala Thr Pro His His Arg
        110   115   120

ccc cag ccc cgt tct gtt ccg ggc tgg gac tct gcc ccc gga gca ccc   480
Pro Gln Pro Arg Ser Val Pro Gly Trp Asp Ser Ala Pro Gly Ala Pro
    125   130   135

tcc cca gct gac atc acc cat ccc act cca gcc cca ggc ccc tct gcc   528
Ser Pro Ala Asp Ile Thr His Pro Thr Pro Ala Pro Gly Pro Ser Ala
140   145   150   155

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His Ala Ala Pro Ser Thr Thr Ser Ala Leu Thr Pro Gly Pro Ala Ala
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   -5   -1 1   5   10

Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
                 15   20   25

Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val
                 30   35   40

Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
                 45   50   55

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln

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Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly

                 80   85   90

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys

                 95   100   105

Lys Asp Ser Ala Val Lys Pro Asp Arg Ala Ala Thr Pro His His Arg

                 110   115   120

Pro Gln Pro Arg Ser Val Pro Gly Trp Asp Ser Ala Pro Gly Ala Pro

         125   130   135

Ser Pro Ala Asp Ile Thr His Pro Thr Pro Ala Pro Gly Pro Ser Ala

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Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln
  -5   -1   1   5   10

agg aaa gtg gtg tca tgg ata gat gtg tat act cgc gct acc tgc cag   144
Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
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ccc cgg gag gtg gtg gtg ccc ttg act gtg gag ctc atg ggc acc gtg   192
Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val
            30   35   40

gcc aaa cag ctg gtg ccc agc tgc gtg act gtg cag cgc tgt ggt ggc   240
Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
            45   50   55

tgc tgc cct gac gat ggc ctg gag tgt gtg ccc act ggg cag cac caa   288
Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
  60   65   70   75

gtc cgg atg cag atc ctc atg atc cgg tac ccg agc agt cag ctg ggg   336
Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly
            80   85   90

gag atg tcc ctg gaa gaa cac agc cag tgt gaa tgc aga cct aaa aaa   384
Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
            95   100   105

aag gac agt gct gtg aag cca gac   408
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Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln
    -5   -1 1   5   10

Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
            15   20   25

Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val

          30   35   40

Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly

     45   50   55

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln

  60   65   70   75

Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly

            80   85   90

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<213> Artificial Sequence

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<210> 13

<211> 53

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer to
     introduce a N-glycosylation site at positions
     289-297 of SEQ ID NO:3 (VEGF-B186)

<400> 13

tcggtaccgg atcatgagga tctgcatggt gacgttgtgc tgcccagtgg cca   53

<210> 14

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR 5' primer

　　for amplification of nucleotides 250 to 567 from

　　Genebank Acc. No. U48801

<400> 14

cctgacgatg gcctggagtg t   21

<210> 15

<211> 50

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR 3' primer

　　for amplification of nucleotides 250 to 567 from

　　Genebank Acc. No. U48801

<400> 15

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<210> 16

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: ` PCR 5' primer

for amplification of nucleotides 1 to 411 from

Genebank Acc. No. U48801

<400> 16

caccatgagc cctctgctcc   20

<210> 17

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR 3'

primer for amplification of nucleotides 1 to 411

from Genebank Acc. No. U48801

<400> 17

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